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Resources Research Inc.

RAPID MICROBIOLOGICAL DETECTION

FINAL REPORT

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RAPID MICROBIOLOGICAL DETECTION

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ABSTRACT

The feasibility of detecting microorganisms rapidly by measuring light emitted when they are introduced into an ATP-dependent firefly luminescence reaction has been established. Instrumentation, consisting basically of a reaction chamber, a photomultiplier system, and an oscilloscope with camera attachment, has been assembled in a manner which permits the observation and recording of the reaction produced by quantities of ATP as low as 2×10^{-4} ug.* Nearly instantaneous, positive responses have been obtained from 16 different microorganisms which included bacteria, yeast, and streptomycetes. Similarly rapid responses have also been obtained from lyophilized bacteria, a commercial preparation of dried yeast, fungus spores, and a soil extract. Bacterial cells in lag phase responded as readily as those in exponential growth. Yeast cells grown for two weeks responded in the same manner as younger cells. Cultures older than two weeks were not tested. A response was obtained from <u>Bacillus subtilis</u> spores when tested after 90 minutes of incubation in a growth medium. Dead cells did not yield a positive response.

Although 40,000 to 50,000 yeast cells were readily detected, the sensitivity of the method will be increased by improving the instrumentation, establishing effective and rapid ATP extraction procedures, and improving the reagents. These are the areas to be investigated more intensively in the next phase of the program.
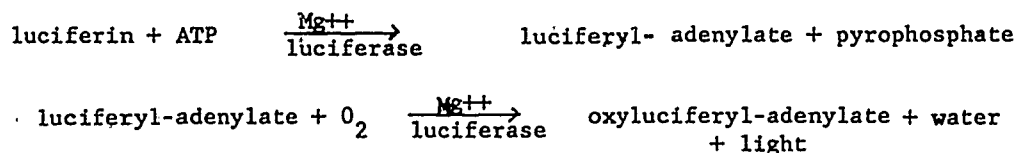
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RAPID MICROBIOLOGICAL DETECTION

I. INTRODUCTION

A need exists for a method which rapidly detects microorganisms under a variety of conditions and circumstances in diverse areas of microbiology. The requirement for a sensitive, simple, and rapid method is probably the greatest where matters of health are concerned and where it is necessary to institute protective measures as quickly as possible. The goal of the present project is to develop a procedure and instrumentation which will permit the rapid detection of microorganisms.

The principle upon which the present approach is based is the observation and measurement of light emission which results when the enzyme and substrate present in firefly lantern extracts are mixed with adenosine triphosphate (ATP). The unique characteristics of this system which make it particularly applicable to microbial detection are: the ubiquity of ATP in living systems, specificity of the assay procedure for ATP, simplicity of procedure, rapidity of procedure, instantaneous response, and greater sensitivity than any other known method for assaying ATP (1,2). In discussing the distribution of ATP, Huennekens and Whiteley (3) point out that although it has been identified in a variety of tissues and cells too numerous to report, "There seems little doubt ... that ATP is the most important 'energy-rich' compound in the entire phyletic sequence, as evidenced by its seemingly universal distribution in animals, plants, and microorganisms." The presence of ATP, therefore, establishes the presence of living organisms. To apply this method to microbial detection "in the field" simply requires a method which enables the selection of microorganisms from among other living organisms. A future effort of the program will be devoted specifically to a study of methods for collecting and

concentrating microbial samples. However, for the present, known micro-
organisms are being used in the laboratory.

A discussion of the biochemistry of luminescence can be found in
Advances in Comparative Physiology and Biochemistry (4). Of the three gener-
al categories (animal, bacterial, firefly), the firefly luminescence system
is the only one which is ATP dependent and thereby applicable to the assay
of ATP. The reactants required for firefly luminescence are the substrate,
luciferin; the enzyme, luciferase; the cofactor, ATP; a cation (usually
magnesium); and oxygen. The overall reaction is an oxidation catalysed by
luciferase, and results in the emission of a quantum of light when the
luciferin molecule is excited and subsequently decomposes. The proposed
mechanism of action is as follows (1,4):

luciferin + ATP $\xrightarrow[\text{luciferase}]{\text{Mg}^{++}}$ luciferyl- adenylate + pyrophosphate

luciferyl-adenylate + O_2 $\xrightarrow[\text{luciferase}]{\text{Mg}^{++}}$ oxyluciferyl-adenylate + water + light

The luciferyl-adenylate is an active form which on oxidation emits light
and becomes oxyluciferyl-adenylate. This, in turn, is bound to the enzyme
(luciferase) resulting in the diminution and cessation of light production.
A number of factors involved in the system have been determined and will
be discussed in the pertinent sections of this report (1,4).

Although it was previously believed that personnel at Resources
Research, Inc. were the first to use the firefly luminescence system to
assay ATP in intact microbial cells, a more recent search of the literature
has shown that the method had been used successfully to study the effect
of ionizing radiation on the ATP content of Escherichia coli (2,5,6). The
results reported in the literature have been substantiated by the work
herein reported. The use of luminescence for the specific purpose of
detecting microorganisms is novel. As a result of the first year's effort,
a fairly broad spectrum of responsive microorganisms has been established.

II. BIOCHEMICAL INVESTIGATION

The fundamental aim of the biochemical investigation was to determine the feasibility of using the firefly luminescent reaction to detect living microorganisms rapidly. This has been successfully accomplished. The procedural details of the method and response have been examined in a survey manner in order to gain some insight into the peculiarities of the method when used specifically as a tool for microbial detection, and to acquire some knowledge of the responses from various microorganisms under a variety of conditions. The results of this screening have been used, and will continue to be used, as guidelines to modify the procedure and the instrument to attain improved sensitivity and reliability from a quantitative aspect. Many parameters have been investigated. Results have been more fruitful in some areas than in others.

A. General Procedure

1. Comparison of Firefly Luminescence to Other Methods of ATP Assay.

A review of various methods for assaying ATP has been made by Strehler and Totter (2). These include chemical separation by chromatography or ion exchange, several enzymatic methods using hexokinase or adenylic deaminase, and the firefly luminescence system. Of the methods used, the firefly luminescence is the most rapid and most sensitive for estimations of submicro quantities of ATP. The greatest sensitivity attainable by each of the methods is as follows: (a) chemical separation, 10-50 ug; (b) ion exchange, 5-30 ug; (c) hexokinase, 2-20 ug; (d) deaminase, 20 ug; and (e) firefly luminescence, $10^{-3} - 10^{-4}$ ug. The greater degree of sensitivity and instantaneous response make the firefly luminescence system the one of choice for microbial detection. Initially, it was the intent of RRI to compare the various methods of ATP assay in the laboratory but sufficient data were found in a continuing search of the literature to make such a comparison unnecessary (1,2).

2. Experimental Approach

Generally, the procedure involves the introduction of standard ATP or ATP-containing material such as microbial cells into a firefly lantern extract. The firefly lantern extract contains luciferin, luciferase, and Mg^{++} as well as other components in the crude material. The substrate, enzyme, and Mg^{++} are sufficient in the presence of O_2, for the assay of ATP. The light emitted when the ATP is added is observed and measured using the instrument system described in Section III of this report. Because the response is instantaneous, the extract must be positioned in front of the light detection system prior to the introduction of ATP into the reaction.

In some instances the commercially prepared firefly lantern extract (which contains $0.05\underline{M}$ $KAsO_4$ and is at pH 7.4) was used as above after reconstitution in deionized water. In other determinations, the reconstituted extract was added to $0.025\underline{M}$ glycylglycine buffer, pH 7.5 or 7.8.

The extract was prepared daily by adding the contents of one vial (approx. 50 mg dry lanterns) to two to five ml of water. The material was then centrifuged for ten min at 1,700 G and the supernatant used as reagent.

The firefly lantern extract was purchased from Worthington Biochemical Corporation; the glycylglycine buffer was prepared from Calbiochem A grade glycylglycine; and ATP for standard determinations was purchased from Schwarz BioResearch, Inc. Deionized water was used routinely for preparing all reagents.

B. Microorganisms Screened

The screening of microorganisms was carried out in two phases.

The first, prior to the availability of an appropriate recording instrument, was carried out visually. The second was performed with the use of the instrumentation described in Section III. The organisms used in the screening program were selected to provide a wide variety of physiological characteristics (Table 1). Included among the organisms is Saccharomyces cerevisiae as a representative fungus and Streptomyces bobiliae as a representative streptomycete. All of the others are bacteria which, as a group, include the following characteristics: Gram positive, Gram negative, strictly aerobic, facultatively anaerobic, spore formers, non-spore formers, various temperature optima, different natural habitats, and other physiological differences such as the ability to produce pigments.

In addition, spore suspensions, lyophilized cultures, dried yeast cells, and a soil extract were tested. It is believed that the microbial spectrum selected provides an adequate variety of physiological types with which the experiment and the instruments can be tested for feasibility.

1. Visual Screening

Only qualitative data were sought in the visual screening of micro-organisms for a response to the firefly luminescence system. Each test was carried out in a dark room after a period of dark adaptation was achieved by wearing fluoroscopic goggles prior to testing the organisms. Cultures of various ages were tested by the following procedure: the organisms were centrifuged and the supernatant discarded. The cells were resuspended in 1.0 ml deionized water and centrifuged again. This supernatant was also discarded. Again, the cells were resuspended in 1.0 ml deionized water and placed in a boiling water bath for ten min. The preparation was then centrifuged and the supernatant was used as the test solution.

The reaction was initiated by adding 0.1 ml of test supernatant to a mixture of 1.0 ml 0.025 $\underline{M}$ glycylglycine buffer (pH 7.5), 0.05 ml

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Table 1

Organisms Tested

Organism	Gram Stain Reaction	Oxygen Requirement	Optimum Temperature (Centrigrade)	Habitat
Arthrobacter simplex	Variable	Aerobic	26-37	Soil
Azotobacter agilis	-	Aerobic	25-28	Soil and water
Azotobacter indicus	-	Aerobic	30	Soil
Bacillus cereus	Variable	Aerobic, facultatively anaerobic	30	Dust
Bacillus subtilis	+	Aerobic, facultatively anaerobic	28-40	Soils
Bacillus subtilis (globigii)	+	Aerobic, facultatively anaerobic	28-40	Soil
Escherichia coli	-	Aerobic, facultatively anaerobic	30-37	Wide in nature, Intestinal Tracts
Micrococcus cinnabareus	Variable	Aerobic	25	Dust
Pseudomonas delphinii	-	Aerobic	25	Plants
Pseudomonas fluorescens	-	Aerobic	20-25	Soil and water
Saccharomyces cerevisiae	+	Aerobic, facultatively anaerobic	30-37	Soil, Plants
Serratia marcescens	-	Aerobic, facultatively anaerobic	25-30	Water, Soil, Milk and Foods
Staphylococcus epidermidis	+	Aerobic, facultatively anaerobic	37	Skin and Mucous membranes
Streptomyces bobiliae	+	Aerobic	37	Soil
Xanthomonas beticola	-	Aerobic	29	Plants
Xanthomonas campestris	-	Aerobic	28-30	Plants

TABLE 2

ATP Assay of Microorganisms By Hot Water Extraction And
Visual Observation

Organism	Medium	Incubation Temperature (Centigrade)	Age of Culture In Hrs.	Response
Saccharomyces cerevisiae	1	37	24	Positive
Saccharomyces cerevisiae	1	24	72	Positive
Saccharomyces cerevisiae	2	37	120	Negative
Azotobacter agilis	2	37	48	Negative
Azotobacter agilis	2	37	48	Positive
Azotobacter agilis	3	37	48	Positive
Pseudomonas fluorescens	2	37	24	Negative
Pseudomonas fluorescens	2	24	120	Positive
Pseudomonas delphinii	2	24	120	Negative
Bacillus cereus	2	37	24	Positive
Bacillus cereus	2	24	48	Positive
Streptomyces bobiliae	2	37	24	Positive
Staphylococcus epidermidis	2	24	72	Positive
Staphylococcus epidermidis	2	37	48	Positive
Arthrobacter simplex	2	37	24	Positive
Bacillus subtilis	2	37	24	Positive
Xanthomonas campestris	2	24	48	Positive
Micrococcus cinnabareus	2	24	48	Positive
Xanthomonas beticola	2	24	24	Positive

1. Difco Sabourand Broth

2. Difco Nutrient Broth

3. Difco peptone 6 gm/l
   Difco yeast extract 3 gm/l
   Difco beef extract 3 gm/l
   Glucose 10 gm/l

of 0.1 $\underline{M}$ MgSO$_4$, and 0.05 ml of firefly lantern extract. Results are presented as positive or negative responses in Table 2.

Approximately 50 observations were made on a total of 12 different organisms. As indicated in Table 2, media, incubation temperatures, and age of cultures varied. Nevertheless, positive responses were observed under one or more conditions with 11 of the 12 organisms visually screened. The one negative culture, Pseudomonas delphinii, yielded a positive response when screened with the more sensitive instrumentation. Experience has also shown that detectable ATP is present in the culture broth which was discarded in the early screening. It is probable that more uniform and consistent positive responses would have been observed if the cells had not been centrifuged from the broth but had been heated and tested in conjunction with it.

2. Electronic Screening

Use of the instrument for screening the response of microorganisms was routinely carried out in the following manner. The electronic components and settings were kept constant except for the sensitivity which was adjusted as necessary to accommodate the wide range of responses. The high voltage was maintained at 2,100 v, and the horizontal sweep rate was maintained at five sec/cm except where otherwise noted in the text.

For testing the unheated samples, the volume of reagents and test material was maintained at 1.0 ml of glycylglycine buffer, 0.1 ml of firefly lantern extract, and 0.1 ml of test material. The test material consisted of whole cell cultures used either directly in the culture medium, or suspended in glycylglycine buffer, or at times both preparations were tested. The buffer suspensions were prepared by centrifuging a pure culture for 10 to 15 min at 1,700 G, discarding the supernatant, and resuspending the cells in 0.2 -0.3 ml of buffer. Some cultures were

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heated in a boiling water bath for ten min. These were tested in 0.2 ml of lantern extract with no additional buffer.

The reagents (except the test material) were placed into a Beckman rectangular, 5 mm, quartz cuvette which was then positioned in front of the phototube. With the reagents in front of the phototube, test material was injected into the reaction chamber through a light-tight port, by using a 20 gauge needle and an 0.25 ml syringe.

Reactions were observed visually on the oscilloscope screen or photographed with the camera system and recorded as a deflection magnitude in millivolts.

Some results of this screening are shown in Table 3 and Figures.1-6.

More than 300 observations were made, but only a representative number of responses are shown in Table 3. The results support the feasibility of the bioluminescent method for detecting a great variety of physiological types of microorganisms. No vegetative culture has failed to respond. However, it is clear that responses are variable. These variations may be the result of variations in cell numbers, in ATP quantities within each type of microorganism, in the amount of ATP extracted, or in some other undetermined factor.

These areas will continue to be intensively investigated.

3. ATP Responses From Microorganisms in Other Than Broth Culture Form

A number of tests were made to determine the applicability of the present procedure and instrumentation to the detection of microorganisms in a form other than a broth culture of vegetative cells. In one test, a soil extract was prepared by adding approximately 500 mg of garden soil to two ml of sterile, distilled water. After standing for 20 min at room temperature, the mixture was centrifuged for one min at 1,700 G and 0.1 ml of the fairly clear supernatant was used as test material. The response, shown in

Table 3

Luminescence Response From One-Tenth Milliliter
of 24 Hour Cultures of Test Organisms

| Microorganism | Deflection(mv) | | Buffer Cell[++] |
| | Broth Culture[+] | | |
	Heated	Unheated	Suspension
Saccharomyces cerevisiae	450	240	-
Bacillus subtilis	-	160	40
Bacillus subtilis (globigii)	320	-	-
Escherichia coli	700	>75	500
Arthrobacter simplex	-	150	-
Staphylococcus epidermidis	-	7500	-
Xanthomonas campestris	-	-	>500
Xanthomonas beticola	45	8	-
Azotobacter indicus	100	0	200
Pseudomonas delphinii	-	-	80
Pseudomonas fluorescens	-	-	>100
Micrococcus cinnabareus	-	-	15
Bacillus cereus	-	80	>40
Serratia marcescens	90	-	-
Streptomyces bobiliae	5	0	-

- not examined

+ Heated;cells and broth heated in boiling
    water bath ten min.
  Unheated; sample taken directly from culture tube.

++ Cells centrifuged from broth and resuspended in
    glycylglycine buffer, pH 7.8
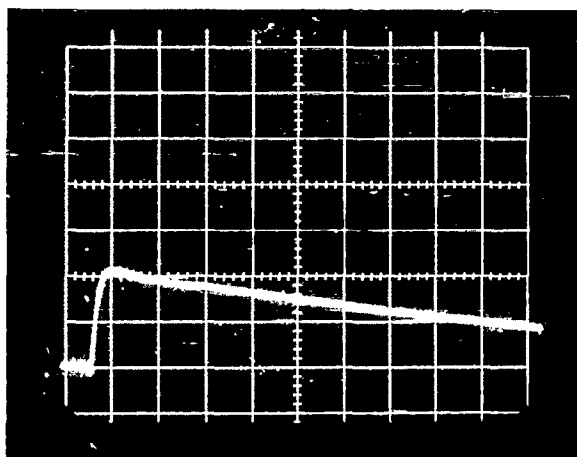
Data are from many separate experiments

Figure 1
Saccharomyces cerevisiae (24 hrs)
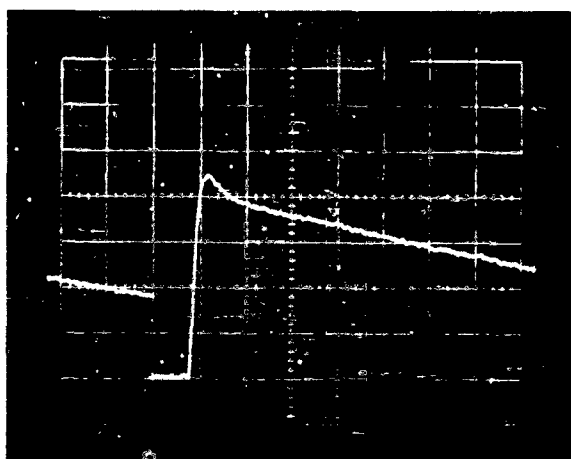Whole cells
Deflection 240 mv
Sensitivity 100 mv/cm

Figure 2
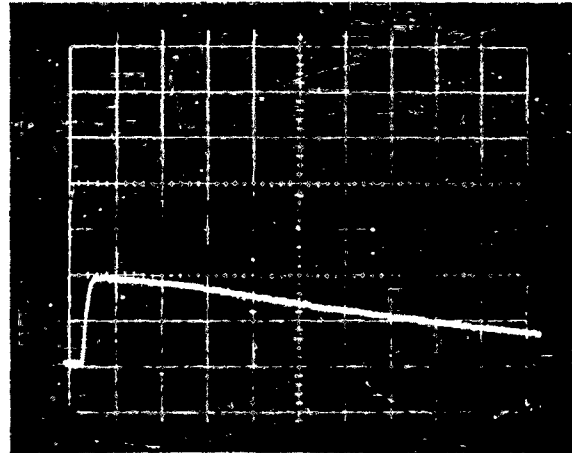Serratia marcescens
Deflection 90 mv
Sensitivity 20 mv/cm

Figure 3
Azotobacter indicus
Deflection 100 mv
Sensitivity 50 mv/cm
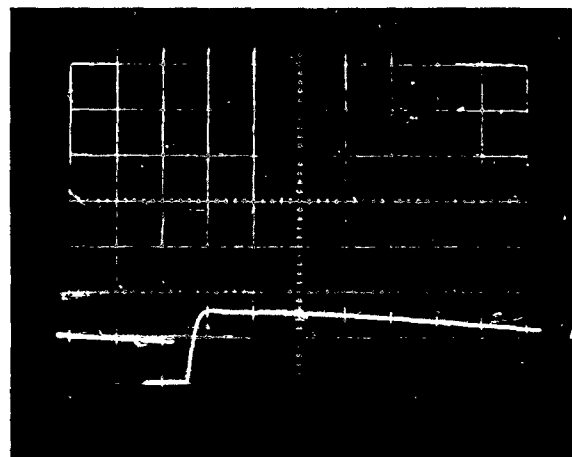

Figure 4
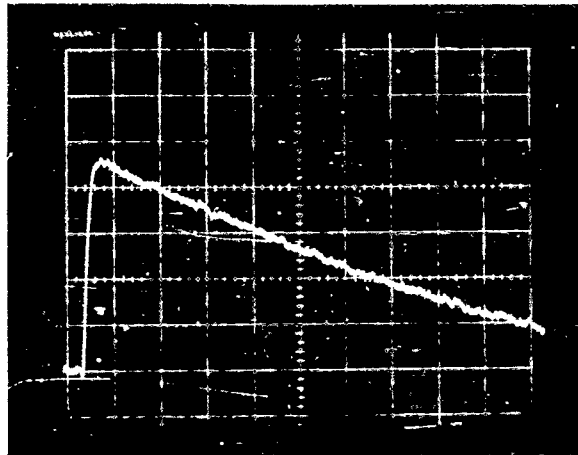Bacillus subtilis (globigii)
Deflection 320 mv
Sensitivity 200 mv/cm

Figure 5
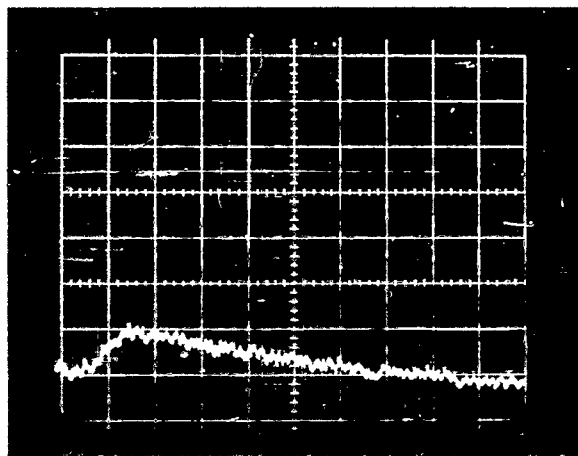Xanthomonas beticola
Deflection 45 mv
Sensitivity 10 mv/cm

Figure 6
Streptomyces bobiliae
Deflection 5 mv
Sensitivity 5 mv/cm

Table 4, demonstrates easily detectable quantities of ATP and probably a large microbial population in the soil extract.

A preparation of commercial (Fleischmann's) dried yeast was also tested. One gram of dry yeast was suspended in five ml water and shaken. One tenth ml of the supernatant was tested. An extremely high response was obtained, indicating the presence of high quantities of ATP in the preparation. The response which is shown in Table 4 and Figure 7 indicates the ability of the method to detect dried microorganisms rapidly.

Lyophilized forms of two organisms were tested. Three mg of the dry Bacillus subtillis (globigii) were placed in one ml deionized water and heated for ten min. One-tenth ml was used as test material, but failed to give a positive response. In a second attempt to test this lyophilized material, 13.5 mg were suspended in 0.4 ml water and heated for five min. One-tenth ml test samples failed to give a positive response.

Lyophilized Serratia marcescens was also tested. Twenty-two mg of material was suspended in 0.44 ml deionized water. The suspension was heated in a boiling water bath for five min and 0.1 ml test sample used. A positive response of 210 mv was obtained (Table 4 and Figure 8).

It can be concluded that microorganisms in forms other than as vegetative growth in culture media can be detected by the luminescence response, since fungal spores, lyophilized cultures, and dried yeast were readily detected. The Bacilli cultures as spore suspensions were resistant and require more intensive investigation. A special discussion of the use of spores as test material is found below.

4. Response From Spore Suspensions

Because of the resistant nature of spores compared to vegetative cells, they are capable of surviving for long periods of time in environments normally unfavorable for microbial growth. When conditions become favorable they germinate into vegetative cells. This unique character-

-14-

Table 4

Luminescence Response From Microorganisms
in Other Than Broth Culture Form

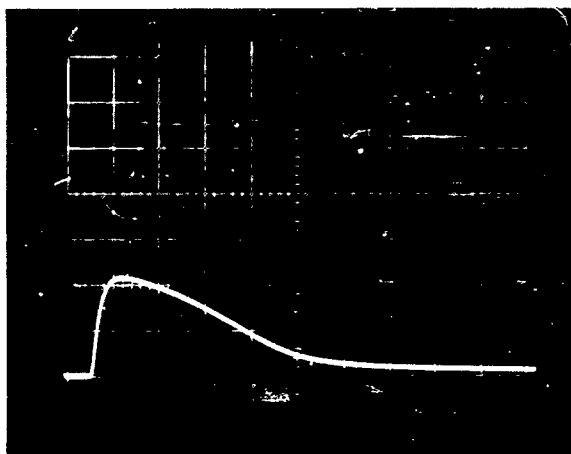| Organism & Form	Deflection (mv)	
	Heated	Unheated
Bacillus subtilis spores	0	0
Soil Extract	-	>450
Dried Yeast (Fleischmann's)	-	4400
Lyophilized Serratia marcescens	210	0
Lyophilized Bacillus subtilis (globigii)	0	0
Fungal spores (Penicillium type fungus)	> 350	58

-   not examined

Figure 7
Dry yeast (Commercial Preparation)
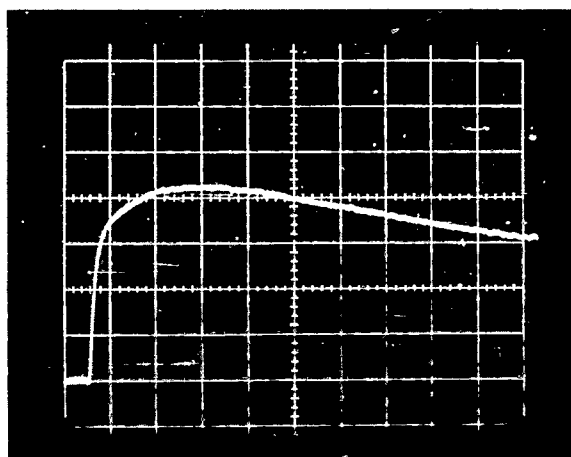Deflection 4,400 mv
Sensitivity 2,000 mv/cm

Figure 8
Lyophilized Serratia marcescens
Deflection 210 mv
Sensitivity 50 mv/cm

istic of spores makes them of special interest since some pathogenic organisms form spores and are often transmitted via the spore stage. Bacillus anthracis, the cause of anthrax is an aerobic bacterium which does this. The Clostridia, which are anaerobic and cause such diseases as tetanus and botulism, are also of this type. Most fungi also form resistant, airborne spores. Therefore, it is of importance to be able to detect microorganisms rapidly in the spore form.

Many fungal spores are formed external to the vegetative portion of the hyphae, in which case pasteurization is not required to prepare a spore suspension. A fungal spore suspension was prepared by flooding a slant of a Penicillium-type fungus with sterile, distilled water. The spores which were not readily wettable, floated on the surface of the water, and were pipetted from the surface into a centrifuge tube. A mixture of the material was tested resulting in the response shown in Figure 9. The suspension was then centrifuged for ten min at 1,700 G and an aliquot of the supernatant which remained turbid was used as the test material. The response obtained from this supernatant unheated was 28 mv. After heating for ten min in a boiling water bath, the response increased about four-fold. The remainder of the material was refrigerated for 60 hrs and a layer of nonwettable spores floated across the liquid surface. These were removed and heated in a boiling water bath for 30 min. The response obtained is shown in Table 4.

Bacillus subtilis spore suspensions were prepared by growing the organisms for about ten days on an enriched agar medium. The growth was then washed from the agar surface with sterile distilled water. Two additional washings of the material by centrifugation were carried out with sterile water. The material was resuspended in distilled water and heated for 30 min at 65°C. This procedure kills the vegetative cells, but does not kill the spores which are formed within the cells. The spore suspension was maintained in the refrigerator at 4°C.
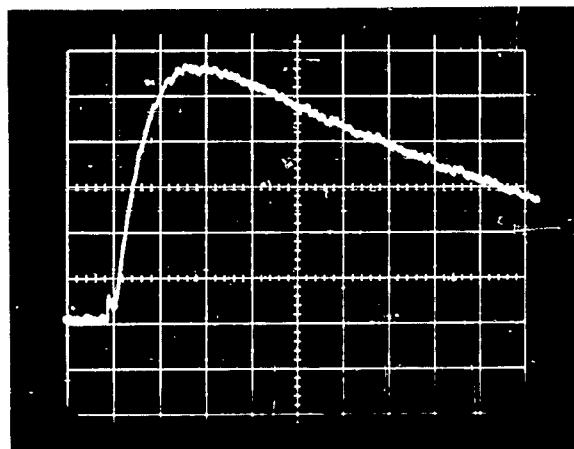
-17-

Figure 9
Fungal spores
Deflection 58 mv
Sensitivity 10 mv/cm

The <u>Bacillus</u> <u>subtilis</u> spores were tested in several ways. A suspension of spores was transferred to a smaller tube and 0.1 ml of suspended spores was used in 0.2 ml of lantern extract. A response of 15 mv was obtained. The spore suspension was then heated in a boiling water bath for 0.5, 2, 5, 12 and 21 min. The responses of all the determinations were about equal until the 21 min heating period. After 21 min no positive response could be obtained. The uniformity of the response after heating was unusual. Subsequent testing suggested that this observed response was not obtained from the spores, but resulted from ATP extracted from the vegetative cells when they were pasteurized. This indicates that <u>Bacillus</u> <u>subtilis</u> spores require some treatment such as sonic disruption or perhaps some kind of solvent extraction of the ATP.

A second approach to the detection of spores consisted of seeding a series of tubes of sterile Nutrient Broth (Difco) and testing the material after various periods of incubation at room temperature. Extraction of ATP was attempted by heating the cells and the broth in a boiling water bath for ten min. On the basis of plate counts, about 80 million spores per ml were introduced into each broth tube. One-tenth of a ml was used for test material. After 3 hrs 40 min a small but positive response of 7 mv was obtained. The broth was not visibly turbid in the growth tube. After 5 hrs 40 min the response was considerably higher (64 mv).

In a similar experiment when the spores were incubated in yeast-dextrose broth at 37°C the responses shown in Table 5 were obtained. It can be seen that compared to deionized water, a marked response was detectable after only 90 min. The response became increasingly more pronounced after two hrs and continued to increase considerably (Figures 10-13). Although an immediate response has not yet been obtained from <u>B.</u> <u>subtilis</u> spores, a reasonably rapid detection of the spores is possible by incubating and following them closely. Nevertheless, efforts will continue toward obtaining a response directly from the spores rather than waiting for the onset of germination.

Table 5

Luminescence Response Obtained From Bacillus Subtilis

Spore Suspension Incubated at $37^{o}C$ for Various

Lengths of Time

Total Time (Incubation + Test - Hrs)	Deflection (mv)
1.5	10
2.0	32
3.0	75
3.5	250
4.5	1400
5.5	2000

Spores incubated in yeast-dextrose broth.

Test material - 0.1 ml of cells in broth heated
    in boiling water bath for ten min.
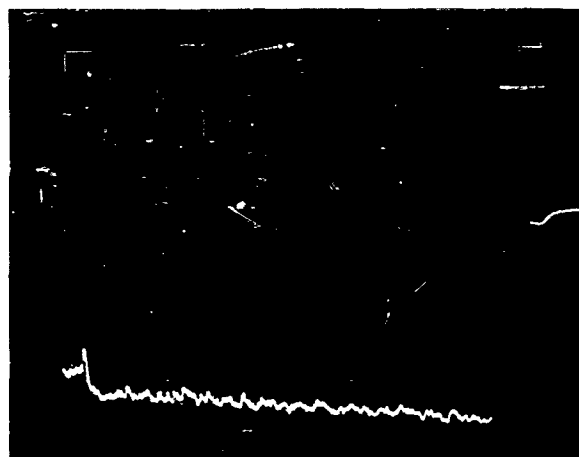
Figure   10
Deionized water
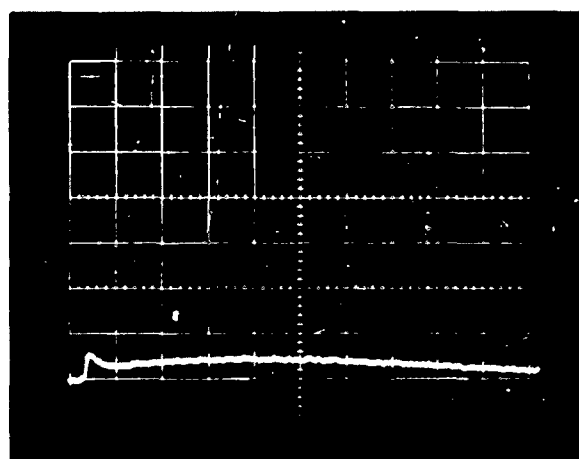Deflection 3 mv
Sensitivity 5 mv/cm

Figure   11
Bacillus subtilis spores in
yeast dextrose broth 1.5 hrs
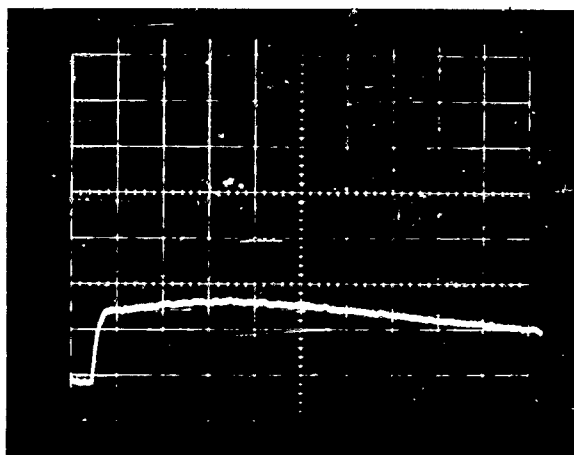Deflection 10 mv
Sensitivity 20 mv/cm

Figure 12
Bacillus subtilis spores in yeast
dextrose broth 2.0 hrs
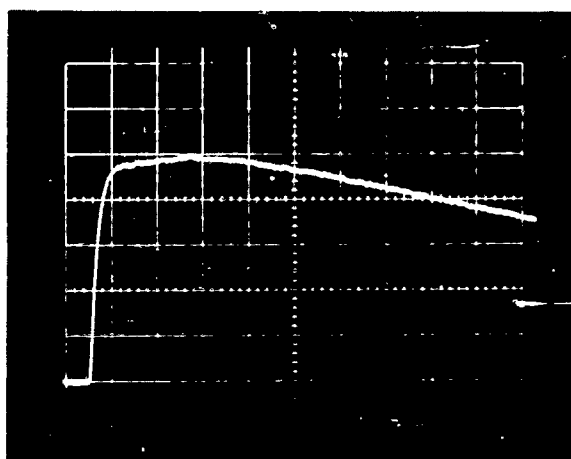Deflection 32 mv
Sensitivity 20 mv/cm

Figure 13
Bacillus subtilis spores in yeast
dextrose broth 3.5 hrs
Deflection 250 mv
Sensitivity 50 mv/cm

C. Sensitivity of the Bioluminescent System

1. Standard ATP Concentrations

Because extract-related variations occur in the luminescence response, measurement of standard ATP solutions of known concentration is essential to an accurate interpretation of the sensitivity obtained from unknown quantities of test material. ATP was measured in a system which utilized 0.2 ml of lantern extract (reconstituted with five ml deionized water per vial, the material was centrifuged for ten min at 1700 G and the supernatant used) and 0.1 ml of ATP solution. The only buffer in the system was the arsenate buffer used in the commercial preparation of the extract. The instrument settings which were utilized were a time constant of five sec/cm, a high voltage of 2100 v and a variable sensitivity to accommodate variations in response.

Some results of the study are presented in Table 6 and Figures 14-18. It can be seen that the response is directly proportional to the ATP concentration when all other factors remain constant. This is in agreement with the results obtained by others using other methods of measuring the luminescence response (1,2,8).

The smallest amount of ATP which could be detected with certainty under the experimental conditions was 2×10^{-4} ug. This sensitivity is better than that reported by Strehler using a quantum counter (2,7). Although Strehler says that 10^{-4} ug can be detected with the quantum counter, the actual amounts reported by him were of an order of magnitude more concentrated. Several factors influence the degree of sensitivity attainable with the present instrumentation and procedure. The first factor is the instrumentation itself. As presently assembled, the noise level begins to mask the signal in a sensitivity range of two mv/cm. It is believed that this can be improved thereby permitting realization of the full capabilities of the components in the system.

Table 6

Response of Standard Solutions of ATP to

The Luminescence Reaction

Concentration ATP - ug	Deflection(mv)
A.	
10^{-1}	4000
10^{-2}	350
10^{-3}	28
B.	
10^{-1}	4800
5×10^{-4}	24
3×10^{-4}	12
2×10^{-4}	6

A and B represent determinations made with
   different extracts and at different times.

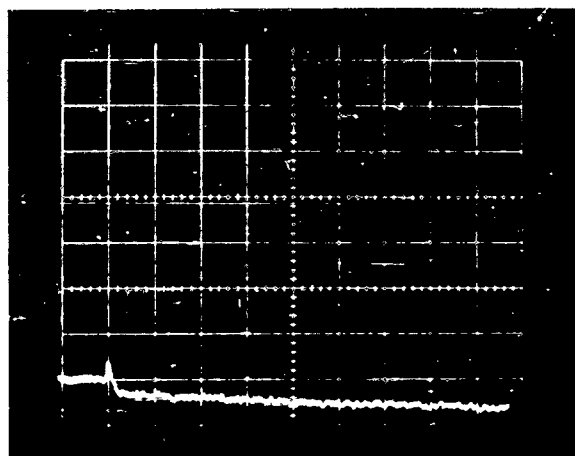Figure 14
Deionized water
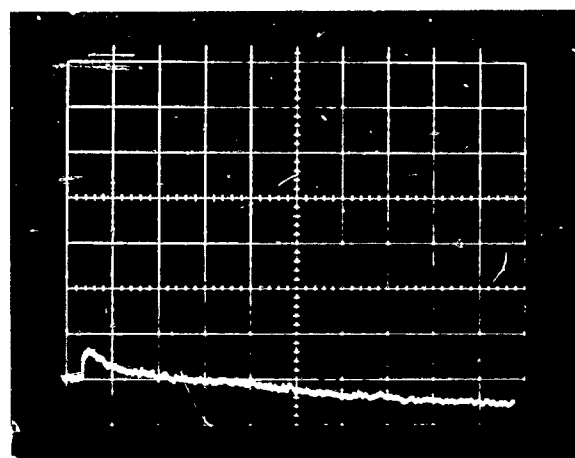Deflection 2 mv
Sensitivity 5 mv/cm

Figure 15
Standard ATP 2×10^{-4} ug
Deflection 6 mv
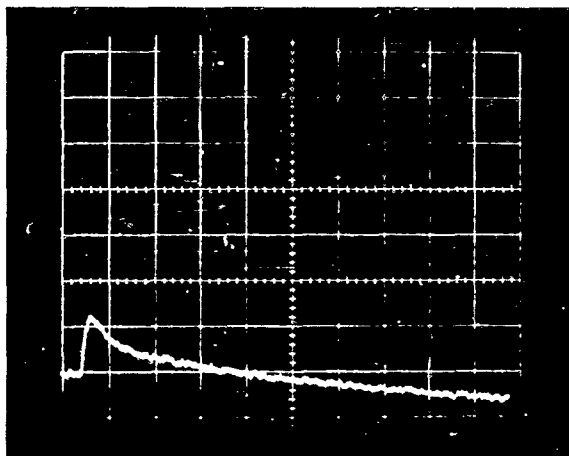Sensitivity 10 mv/cm

Figure 16
Standard ATP 3×10^{-4} ug
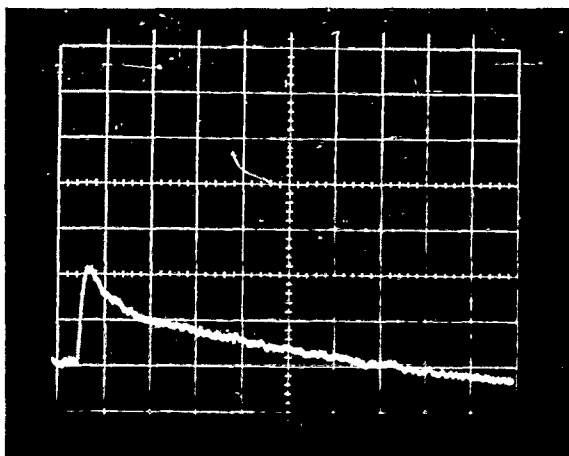Deflection 12 mv
Sensitivity 10 mv/cm

Figure 17
Standard ATP 5×10^{-4} ug
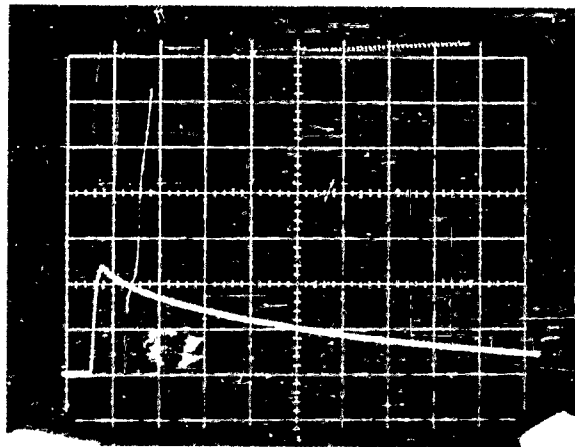Deflection 24 mv
Sensitivity 10 mv/cm

Figure 18
Standard ATP 10^{-1} ug
Deflection 4,800 mv
Sensitivity 2,000 mv/cm

The second factor which presently limits the sensitivity is the crude lantern extract itself. Each preparation of lantern extract has a level of inherent light which varies in intensity from several mv to several hundred mv. Currently, the source of this light is unidentified. The majority of responses fall between 30 and 100 mv. The inherent light can be nulled out electronically. However, it has been found that when the reagents are mechanically disturbed in some manner such as by mixing with deionized water, or by stirring with air introduced from a syringe beneath the surface of the reagent mixture, a light is emitted in a way which results in a peak deflection (Figures 10 and 14). This is probably due to changes in the amount of light directed to the instrument, and caused by reflection and refraction resulting from the liquid movement. On visual observation this response appears to be the same as the response obtained from the introduction of low concentrations of ATP into the reagents. However, photographically, the response exhibits a very sharp decrease with time and results in considerably less area under the curve than the responses obtained from ATP. (Figure 15). Under the present conditions it is essential to test distilled water or sterile broth routinely for comparison with the unknowns.

A number of possibilities exist for improving the overall sensitivity of the system by decreasing the inherent light in the crude extract. It is possible that the interfering material is associated with particulate matter in the extract. There is some evidence which shows that removal of particulate matter reduces the amount of inherent light. As reported in Quarterly Progress Report No. 2, filtration of the extract through a Millipore membrane filter decreases the inherent light, although the response to ATP also decreases. A second possibility for reducing the light would be to use purified luciferin and luciferase. Initial efforts

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to prepare these in purified form were also reported in Quarterly Progress Report No. 2. Because the light could be nulled out electronically and the response from cells being screened was generally much higher than the inherent light, no intensive investigation of purifying the enzyme and substrate was pursued. Efforts may have to be resumed along these lines in order to obtain maximum sensitivity from the procedure. One remaining method of controlling the interference from any residual inherent light would be to eliminate the mechanical disturbance created within the mixture. This might be achieved by designing a cuvette which would allow mixing without agitation of the mixture. It is quite likely that a combination of approaches will be required.

2. Sensitivity Using Microbial Cells

Achievable sensitivity of the luminescence response is influenced by a number of factors. Some of these factors are: the type of microbial cells, variations of age, state (vegetative or spore), and growth media. Another important factor is the kind and degree of extraction procedure to which the ATP within the cells is subjected. Influence of the test system, i.e., the concentration of extract, the age of extract, and possibly the particular preparation of extract (there are some data which show that variations exist between extracts purchased at different times from the same commercial source) are also factors. Still another parameter is the sensitivity of the instrumentation.

It becomes obvious that all of these variables require investigation at some stage of the research. It also becomes obvious that a choice must be made to establish a starting point. Therefore, in the present investigation, Saccharomyces cerevisiae was selected as the organism to examine first for a number of reasons. It grows well, responds to the

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system reasonably well, contains spores, and is relatively resistant to cell rupture.

A preliminary sensitivity study based on culture dilution rather than determination of cell numbers was reported in Progress Report No. 3. The results indicated that responses were obtained from dilutions beyond the point where cells might still be expected to be present. Subsequent experiments relating to enzyme concentration and the emission of a certain amount of inherent light from the crude extract emphasized the need to interpret the results cautiously. Although the responses appear to be real, the absence of a distilled water control determination for comparison introduces a degree of uncertainty.

In other efforts to evaluate the sensitivity of the luminescence response, actual cell numbers were calculated. The age of the culture was also maintained constant, 20-24 hour cells being selected for the initial studies. Growth was always in Sabouraud Liquid Medium (Difco). The reagents used for a comparison of cell responses were 0.2 ml of firefly lantern extract (Worthington prepared as described above) and 0.1 ml of test material. The reaction was carried out in five mm Beckman quartz cuvettes at 2,100 v. Both visual and photographic observations were made. Cell numbers were determined by plate counts and by use of a standard turbidimetric curve determined prior to the initiation of the study.

Initially, whole cells in broth were used as test material. They were centrifuged and resuspended in deionized water. Dilutions were made from the resuspended cells in deionized water. The supernatants were also tested for response to the firefly luminescence system. Approximately 90 observations were made on the cells grown and tested in this manner. Representative responses obtained from whole, untreated cells and super-

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natants are shown in Table 7.

Several conclusions can be drawn from an examination of the results. Under the test conditions, 40,000 to 50,000 cells could be detected with a reasonable degree of replication. While these results represent a fair degree of sensitivity, the results also showed that most of the ATP measured was present in the broth supernatant. Therefore, it seems reasonable that if some type of extraction technique is utilized, the sensitivity of the test can be improved. The results also demonstrated the influence of the firefly lantern extract on the absolute values obtained as peak deflection in millivolts. It can be seen that extract which yielded a higher response to standard 10^{-1} ug ATP solutions usually yielded a higher response to smaller numbers of cells. Results such as these demonstrate the necessity of determining the response to known concentrations of ATP and the desirability of obtaining lantern extract which yields a maximum response. The first aspect, routine determination of standard ATP responses, is being carried out with daily determinations. The second, that of obtaining extracts of high responsive yield, is presently dependent upon chance, but will be discussed with the manufacturer. However, related to this problem is the one discussed earlier, that is, the preparation of particle-free extracts or the use of purified enzyme and substrate. A greater degree of uniformity might be achieved in this way. One other conclusion to be drawn from the sensitivity studies is that resuspension of cells without any ATP extraction results in inconsistent responses when cell numbers and peak deflections are compared. It is conceivable that the inconsistency of the response is related to the length of time between the suspension in water and the testing of the material. Greater consistency is obtained when hot water extraction is used to obtain the ATP from diluted cultures (Table 8).

Table 7

Test Sensitivity as Determined by Responses From Unheated
24 Hour Saccharomyces Cerevisiae Broth Cultures

No. of Cells in Test	Deflection (mv)	
	Test	Std - 10^{-1}ug ATP
A.		11,000
500,000 in broth	240	
Supernatant*	300	
500,000 resuspended in H_2O	40	
250,000 " " "	300	
100,000 " " "	75	
50,000 " " "	80	
B.		9,000
450,000 in broth	190	
Supernatant*	180	
450,000 resuspended in H_2O	36	
225,000 " " "	15	
90,000 " " "	10	
45,000 " " "	10	
C.		4,000
860,000 in broth	104	
Supernatant*	90	
860,000 resuspended in H_2O	250	
430,000 " " "	180	
172,000 " " "	64	
86,000 " " "	100	
43,000 " " "	70	

* Supernatant from first group of cells tested in each series.

Table 8

Effect of Heating on Luminescence Response
From 24 Hour Saccharomyces Cerevisiae Culture

| Culture Resuspended | Deflection (mv) | |
in Water	Unheated	Heated
Undiluted	75	450
Diluted 2X	70	130
Diluted 10X	17	28

0.1 ml of each used in test.

Culture centrifuged from broth and resuspended

in deionized water.

D. Extraction of ATP from Microbial Cells

In the interest of developing a simple and rapid procedure, whole, untreated cells were investigated during the screening studies and the initial sensitivity studies in which the instrumentation was utilized for observing and measuring the luminescence. Nevertheless, there seems little doubt that some method of ATP extraction enhances the response from microbial cells. This observation was made by Billen et al. (5) and Billen and Volkin (6) in a study of the effects of ionizing radiation on the ATP content of Escherichia coli, as well as by Strehler (9) in a study of photosynthesis in Chlorella. A number of observations during the current program also support the idea that extraction would be helpful.

In the pre-instrumentation phase of this program, a number of extraction methods were used to prepare test material which would evoke a visual response. The extraction methods which were attempted, but which resulted in negative responses from Escherichia coli, were: cold water extraction from an acetone powder, and lysozyme in the presense of ethylenediaminetetraacetic acid. Positive responses were obtained when extraction was carried out with five per cent perchloric acid for one min or with hot water. Because the hot water extraction involved only boiling in a water bath for a period of time (teh min was used first), and was the simplest and most direct procedure, it was used as the method for preparing material for the visual observations reported in an earlier section.

The desirability of increasing the test sensitivity led to a further examination of the effects of hot water extraction. Several time-response determinations were made using 20-24 hr cultures of Saccharomytes cerevisiae and heating them for various periods of time. The response was determined by using 0.1 ml aliquots of the heated cells in broth, 0.1 ml

aliquots of supernatant, or 0.1 ml samples of cells resuspended in water and heated. Two-tenths ml of firefly lantern extract were used as reagent. The test was carried out at 2,100 v and responses observed both visually on the oscilloscope and recorded photographically.

The type of responses obtained are shown in Tables 8 and 9. It is obvious that heating yields greater amounts of measurable ATP and more consistent results. The ATP is not adversely affected by the ten min heating period. Heating was incorporated into the daily routine.

E. Response From Cells in Lag and Exponential Growth

In order to examine the response from cells which are in lag or exponential growth phases, a 24 hr broth culture of Escherichia coli was centrifuged from the growth medium. The supernatant was discarded. The cell pack was then resuspended in sterile yeast-dextrose broth and again the culture was centrifuged and the supernatant discarded. The cells were again resuspended in three ml of sterile broth and 0.1 ml of the suspension was used to inoculate each of a series of tubes containing three ml yeast-dextrose broth. In this way no residual ATP was carried from the initial broth culture supernatant. All of the inoculated tubes were refrigerated at $4^{O}C$ for two hrs to induce lag. After the refrigeration period, the tubes were removed, and the optical density was determined using a B. & L. Spectronic 20. One tube was used for testing, and the others were incubated at $37^{O}C$. Individual tubes were removed from the incubator periodically and the O.D. of each was determined prior to using it for test material. Each tube (cells and broth) was heated for ten min in a boiling water bath prior to using 0.1 ml of each for testing. The results are shown in Table 10. The O.D. of the initial tube was zero.

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Table 9

Effect of Heating on Luminescence Responses From
24 Hour Culture of Saccharomyces Cerevisiae and

10^{-2} ug ATP

Treatment	Deflection (mv)	
	Culture	10^{-2} ug ATP
Untreated	50	580
Washed Culture-Resuspended in H_2O	25	
Resuspended Culture - Heated* 30 seconds	130	460
Resuspended Culture-Heated 1 min	600	490
Resuspended Culture-Heated 2 min	880	440
Resuspended Culture-Heated 5 min	1500	450
Resuspended Culture-Heated 10 min	1700	470

* Cultures were placed in test tubes and suspended
  in a boiling water bath.

  Test   culture was 0.1 ml containing 960,000 cells.

Table 10

Response of Escherichia Coli in Lag and
Exponential Growth

Growth Phase	Optical Density of Culture	Approx. No. of cells (X 10^8)	Deflection (mv)
Lag	0.000		28
Lag	0.105	2.2	280
Transitional	0.190	2.7	625
Exponential	0.230	4.0	800
Exponential	0.315	6.0	-
Exponential	0.370	7.4	1,000

Test material - 0.1 ml of cells in broth

heated in a boiling water bath for ten min.

In spite of the lack of detectable turbidity, a slight positive luminescence response was obtained. As growth began, as indicated by the increase in turbidity, the response in the luminescence reaction increased correspondingly. A growth curve indicates that the cultures in the last two determinations were in exponential growth, whereas in the first two they were in lag. They were coming out of lag and entering the exponential phase when the third determination was made. It appears that the stage of growth does not greatly affect the ATP response except as a direct reflection of the number of cells present in the test culture. Until the quantities of ATP per cell can be determined precisely, it will not be possible to establish the effect on individual cells more accurately.

Nevertheless, studies, reported below, with Saccharomyces cerevisiae of various ages show the response from cultures in stationary and death phases (Table 11). It can be concluded that an easily observable response can be obtained from microbial cells in all growth phases.

F. Response From Cells of Various Ages

Tubes of Sabouraud Broth were seeded with Saccharomyces cerevisiae and permitted to grow at room temperature. After they had grown for various periods, the cells were heated directly in the growth medium in a boiling water bath for ten min to extract the ATP. One-tenth ml aliquots were tested for response to the luminescence system containing 0.2 ml crude lantern extract. Plate counts were also made prior to heating the cultures. The responses and numbers of cells from cultures of various ages are shown in Table 11. It can be seen that the response from the older cultures was less than from the younger cultures. The

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Table 11

Response From Cultures of Saccharomyces
Cerevisiae of Various Ages

Age of Culture (days)	Approximate Total Cell Numbers in Broth	Deflection (mv)
4	1,030,000	2,500
5	1,220,000	2,000
7	1,320,000	2,500
11	860,000	1,300
12	250,000	1,700
13	1,200,000	1,200
14	460,000	900

Cells were grown in Sabouraud Broth

Test material - 0.1 ml of cells in broth heated

    in boiling water bath for ten min. Represents

    ATP in supernatant and extracted from cells.

cell numbers were, with one exception, also fewer in the older cultures. Simple correlation between cell numbers and response is not readily apparent. A number of other factors, such as the amount of ATP in the broth as a result of cell lysis, and the production and stability of ATP with time, must also be considered. It can be concluded that cultures two weeks old (and probably older ones) will respond in the luminescence system.

G. Dead Cells

Because ATP is associated with living cells, it is reasonable to assume that cells which have been killed will no longer yield a response to the luminescence reaction. However, it was believed to be essential to investigate the response obtained from dead cells. This was done by exposing a 24 hr culture of Escherichia coli in Nutrient Broth to two per cent Lysol for about five hrs. Lysol contains soap, orthohydroxydiphenyl, alcohol, pine oil, propylene glycol, and glycerol. The Lysol was then removed by centrifuging the cells. The cells were washed five more times to remove traces of Lysol prior to ATP extraction, resuspended in de-ionized water, heated for 15 min in a boiling water bath and 0.1 ml of material tested. Lysol-exposed cells were also used to inoculate tubes of sterile broth. The response from a number of untreated cells equal to the number of treated cells was 700 mv. No response was obtained from the killed cells. No growth resulted from the inoculation of the broth, indicating the effectiveness of the disinfectant.

A similar experiment was carried out using a 24 hr culture of Saccharomyces cerevisiae, exposed to two per cent Lysol for 20 hrs. The

results were the same with the yeast as with the bacterium. A high

response was obtained from cells which were not exposed to Lysol and a

negative response resulted from the killed cells. No growth was observed

on slants inoculated from the dead culture. Concentrations of Lysol from

0.00001% to 0.01% were found uninhibitory to a response from 10^{-1} ug ATP.

Since these concentrations were probably in excess of any residual Lysol

after the cells were washed, it seems certain that dead cells will not

respond in the luminescence system.

H. Reproducibility of Response

In order to determine if the same number of the same organisms

grown under the same conditions would respond in a similar manner in the

luminescent system, the following determinations were made. A 24 hr

culture of Saccharomyces cerevisiae was divided into four equal portions.

Each aliquot was then treated in an identical manner. The response of

the initial culture was determined. After the cells were centrifuged out

of each of the four aliquots, the response of each supernatant was

measured. Each culture was resuspended in deionized water and the

responses again measured. Finally, each culture was heated in a boiling

water bath for five min and the response of each was again measured. The

results are shown in Table 12.

It can be seen that the reproducibility is quite good. The only

exception is the heated culture A, which responded about three times as

much as the other cultures. It is conceivable that this culture con-

tained cells in excess of the other cultures as a result of unequal divi-

sion of cells initially. When cells clump together, an unequal distribu-

tion in a case like this is not uncommon. Because of the effectiveness

of the heating extraction, it is at this point in the procedure where an

Table 12

Reproducibility of Luminescence Response From Equal Aliquots
of the Same Initial Saccharomyces Cerevisiae Culture

Culture	Deflection (mv)
Initial Culture-Untreated	230
Supernatant A	240
B	240
C	230
D	240
Cells Resuspended in H_2O	
A	84
B	70
C	90
D	60
Resuspended Cells Heated 5 min	
A	1500
B	450
C	650
D	550

excess of cells, and consequently excess ATP, would be most noticeable.

In general, the method offers reasonably reproducible results.

III. INSTRUMENTATION

The basic purpose of any instrumentation for microbial detection using the firefly luminescence reaction is to provide a means whereby small quantities of light can be quantitatively observed and recorded. In addition, the instrumentation should be trouble-free and simple to use. As presently designed, the light detection system meets these criteria and is also flexible because most of the components are stock items. The flexible nature is desirable, since further changes and improvements are anticipated. A diagrammatic representation is shown in Figure 19. Part of the assembly consists of a composite sensing and reaction chamber which contains an RCA 7265 photomultiplier tube and a rotary cylinder mounted in a block of aluminum in a manner which permits removal of the reaction chamber without exposing the phototube to light. A section of the cylinder wall is cut out to accommodate a standard ten mm or five mm Beckman DU rectangular cuvette. Immediately above the cuvette holder is a small injection port sealed with a replaceable light-tight rubber plug. The entire unit is painted black to reduce light reflection.

An accessory box is also part of the assembly. It contains conventional circuitry for providing the required voltage for the phototube. It also contains a resistance-capacitance network to reduce the signal bandwidth, which restricts the noise level and enhances the detection of smaller signals. The peak-to-peak noise is about one mv across an approximate one megohm load consisting mostly of the input impedance of the oscilloscope. A further component of the accessory box is circuitry to balance out the phototube dark current, thereby permitting the use of high gain in the oscilloscope. A schematic of the phototube and associated circuitry is shown in Figure 20.
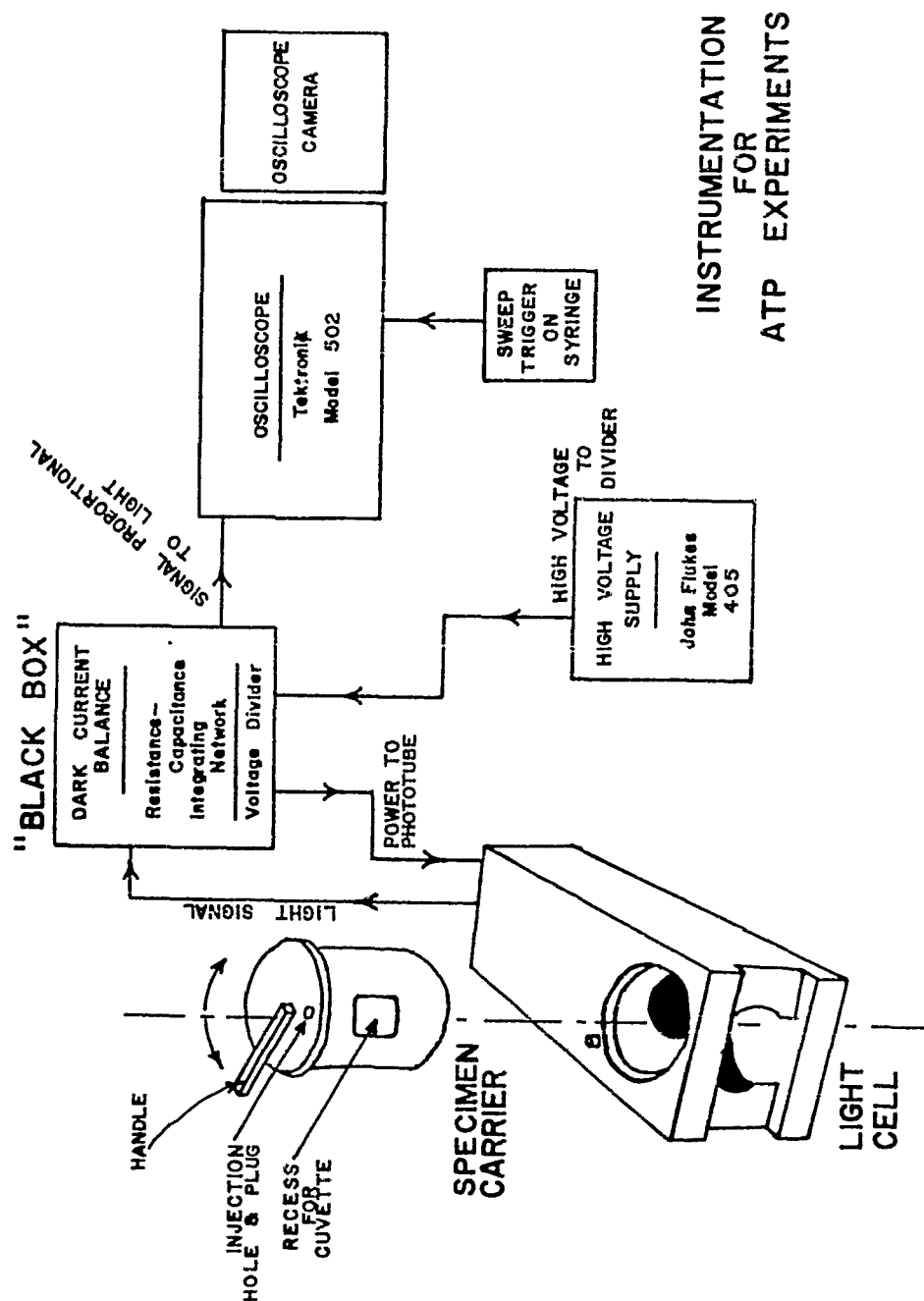
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INSTRUMENTATION
FOR
ATP EXPERIMENTS

Figure 19

OSCILLOSCOPE
CAMERA

OSCILLOSCOPE
Tektronix
Model 502

SWEEP
TRIGGER
ON
SYRINGE

SIGNAL PROPORTIONAL
TO LIGHT

"BLACK BOX"

DARK CURRENT
BALANCE

Resistance—
Capacitance
Integrating
Network

Voltage Divider

HIGH VOLTAGE
TO DIVIDER

HIGH VOLTAGE
SUPPLY

John Flukes
Model
405

POWER TO
PHOTOTUBE

LIGHT SIGNAL

HANDLE

INJECTION
HOLE & PLUG

RECESS
FOR
CUVETTE

SPECIMEN
CARRIER

LIGHT
CELL
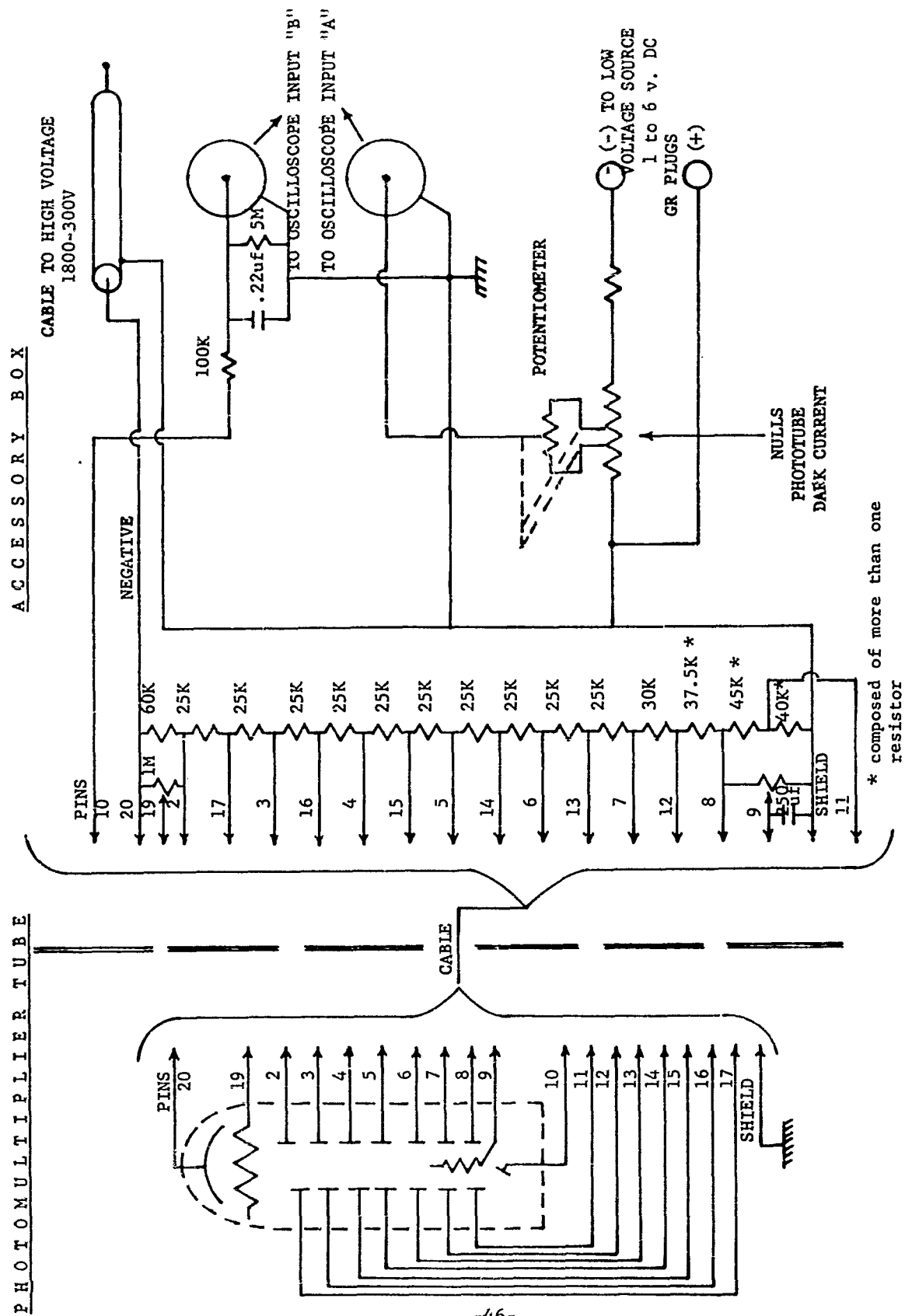
SCHEMATIC OF PHOTOTUBE AND ASSOCIATED CIRCUITRY

Figure 20

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The high voltage source is a well regulated, variable instrument manufactured by John Flukes, Model 405. It also has a reference voltage available which can be used to power the dark current balance circuitry.

The oscilloscope is a Tektronix Model 502 Dual Beam with a maximum sensitivity of 200 uv/cm of beam deflection which will allow an increase in system sensitivity by decreasing the bandwidth or directly reducing the noise level. There is a multiple switching arrangement at the scope input which makes it convenient to adjust the system zeros and balances. The differential input to the scope provides an ideal point to balance the dark current output of the phototube. A triggering system which is used to initiate the horizontal sweep has been added to the oscilloscope.

The response to the firefly luminescent system is recorded with a Tektronix C-13 Polaroid camera which mounts directly onto the front of the oscilloscope. Polaroid roll film, type 47, speed 3000 is used in the camera. The lens is an Oscillo-amator f/4.5. Figure 21 shows the instrumentation without the camera.

To observe and record the reaction, the cuvette containing the necessary reagents is positioned in the cuvette carrier without exposing the phototube. Rotation of the carrier positions the cuvette in front of the phototube. The unknown, or ATP sample, is then added through the injection port. The rotary cylinder design utilizes non-reflecting curved paths to prevent light from penetrating from the exterior of the accessory box.

The present instrumentation is simple to use and facilitates the detection of small quantities of ATP. It is believed that its sensitivity
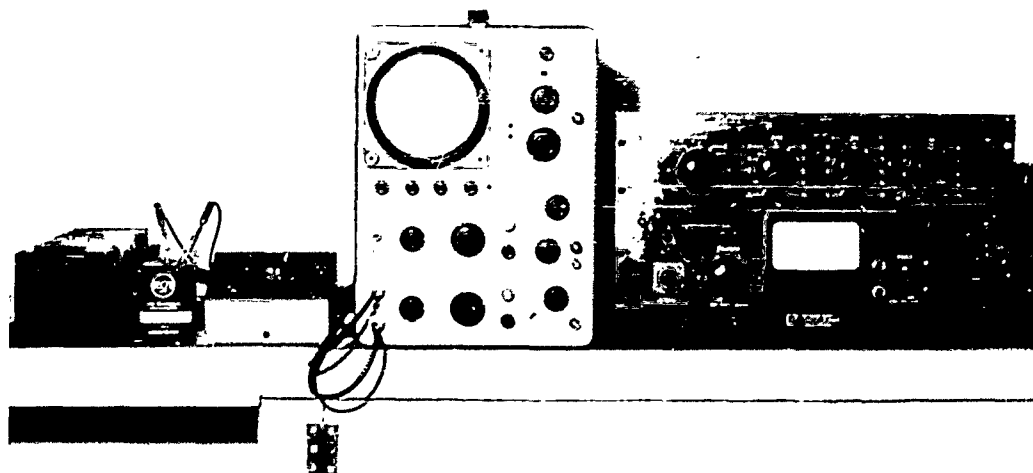
-47-

Figure 21

Light Detection System

can be further increased, but the inherent light in the extract will have
to be eliminated to take full advantage of the instrument's potential.

IV. FUTURE INVESTIGATION

Many areas of future investigative efforts have been mentioned above in conjunction with evaluation of results. The fundamental purpose of all future investigation will be two-fold. First, efforts must be made to improve the sensitivity although the present method is more sensitive than any previously described. Secondly, the procedure must be standardized in a way which will permit the rapid detection of microorganisms regardless of the form in which they are found. Overall, the program for the second year, as proposed initially, will be carried out. This included the development of a "breadboard layout" of a functional device, the testing of the equipment and procedure under field conditions, the use of pathogenic microorganisms in conjunction with a government facility, and a general effort to optimize sensitivity, simplicity, and rapidity. Initially, a study of ADP-ATP ratios in whole cells and extracts was envisioned. Because it appears that extraction of ATP will be essential for maximum sensitivity, a comparison of cells and extracts would contribute little toward achieving the goal of the project. The enzymatic conversion of ADP to ATP might have application and will be considered.

Laboratory efforts will be made to improve sensitivity and magnitude of response by several means. The problem of obtaining firefly lantern extracts of uniform response was discussed earlier. It will be necessary to reduce the inherent light by possibly removing at least a portion of the particulate matter. It might also be possible to purify the enzyme, luciferase, and the luciferin substrate to achieve the desired degree of sensitivity and uniformity. Further efforts are anticipated in an investigation of the application of the anaerobic flash phenomenon in which the initial reaction is allowed to proceed anaerobically and the subsequent introduction of

oxygen results in a response approximately ten times that obtained when the overall reaction proceeds aerobically. McElroy (1) reports that phosphates or Coenzyme A added after the ATP has been added to the reaction increases the response. These factors should also be investigated for possible application in the microbial detection system.

Also to be considered is the value of using the entire area under the response curve rather than using the initial deflection as the sole measure of response. The advantage of using the entire curve, and, indeed the danger of using only peak magnitude, is evident when the response from deionized water is compared (Figure 14) to some of the responses from lower concentrations of ATP (Figure 15) This aspect of the procedure offers good promise of increasing sensitivity and improving reproducibility.

The instrumentation will receive careful evaluation. Changes which will permit a more efficient detection of the light emitted are under consideration. The most immediate approach is to add reflector material to the rear of the reaction chamber in a way which would direct and concentrate the light on the photomultiplier tube. Also to be considered is the use of an integrating sphere for carrying out the response. This innovation may permit more efficient light detection than the present system yields.
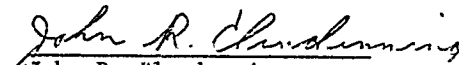
As these areas are investigated and the problems presented by each are solved successfully, the ATP method of microbial detection will be even more rapid, simple, and practicable. Prospects seem very good for the ultimate development of a simple field kit much as visualized in the original proposal for this research program.

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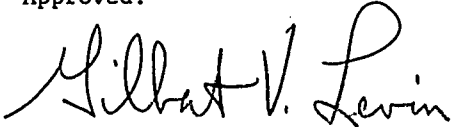
April 30, 1963

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